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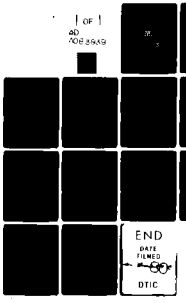

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Over the past year, this laboratory produced numerous advances in the understanding of HLA-D region immunogenetics. We were major participants in the Eighth International Histocompatiblity Workshop. We identified a number of new HLA-D specificities and furthered our understanding of the interaction of HLA-D and HLA-DR. We have continued to develop primed lymphocyte typing and methods of lymphocyte cloning. A new method of analyzing homozygous typing cell data was developed along with additional computer techniques.

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We have continued to develop our understanding of the effect of interferon on the immune system as it relates to the major histocompatibility complex mechanisms. Finally, this laboratory is organizing the Second International Conference on the Primed Lymphocytes in conjunction with the National Institutes of Health and Naval Medical Research Institute.

OFFICE OF NAVAL RESEARCH

Contract N000-14-77-C-0747

Task No. NR 207-101

TECHNICAL REPORT NO. 3

HISTOCOMPATIBILITY TYPING

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28 March 1980

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A. ABSTRACT

~~Over the past~~ year, this laboratory produced numerous advances in the understanding of HLA-D region immunogenetics. ~~We~~ were major participants in ~~the Eighth International Histocompatibility Workshop.~~ ~~We identified~~ a number of new HLA-D specificities has been identified and ~~furthered~~ our understanding of the interaction of HLA-D and HLA-DR has been furthered. ~~We have continued to develop~~ primed lymphocyte typing and methods of lymphocyte cloning are being developed. A new method of analyzing homozygous typing cell data was developed along with additional computer techniques.

~~We have continued~~ to develop our understanding of the effect of interferon on the immune system is being studied as it relates to the major histocompatibility complex mechanisms. Finally, this laboratory is organizing the Second International Conference on the Primed Lymphocytes in conjunction with the National Institutes of Health and Naval Medical Research Institute.

B. INTRODUCTION

The following technical report summarizes the third year's progress on ONR contract N000-14-77-C-0747 "Histocompatibility Typing". This contract was initiated on September 15, 1977 for the purpose of developing HLA-D histocompatibility testing to permit the potential availability of non family member bone marrow transplant donors.

C. BACKGROUND

Military personnel are at far greater risk of contact with bone marrow toxic agents than the civilian population by virtue of working in hazardous areas. The necessity of using various toxic fuels and chemicals as well as potential exposure to irradiation produce a hazard which requires the availability of bone marrow transplantation for treatment of aplastic anemia from whatever cause. Bone marrow transplantation in cases of aplastic anemia has become an accepted form of treatment and is currently funded in civilian institutions by health insurance plans. However, at this time, the only long term survivors treated with this form of therapy have occurred when HLA identical siblings have used as the marrow donor. As only one third of the potential transplant recipients have matched sibling donors it is necessary to identify methods of tissue matching which will allow the use of unrelated donors. Improved methods of tissue typing would then allow a much broader use of bone marrow transplantation. Further, the identification of those specificities which must be closely matched in donor and recipient versus those specificities with little clinical consequence will broaden the number of potential transplant donors.

It is clear that the success of transplants is greatest with the most compatible grafts, and is much less successful with poorly matched tissues. We have therefore initiated a program of tissue typing in order to define the essential components of graft rejection and to seek ways to alter the body's natural rejection mechanisms.

At the present time tissue typing is essential for all current transplant programs. However, it is clear that typing of histocompatibility antigens must be extended and further refined to fulfill its potential as a major clinical tool. To outline the complexity of the HLA tissue typing system, it now appears that there are at least six gene loci which control histocompatibility antigens on the human cell surface which have been called HLA-A, -B, -C, -D, -DR and MB and code for approximately 85 currently identified unique antigens. These histocompatibility antigens are responsible for the rejection or acceptance of tissue grafts. Of these, the HLA-D region loci are the least understood; however, they seem to play a dominant role in the acceptance or rejection of the tissue graft and play a major role in control of the immune response. The research that we have initiated is directed toward the problem of examining this HLA-D genetic region, defining the HLA-D locus antigens, and cataloging them in such a manner that individuals could be easily typed for these most important determinants. It is estimated that the twelve currently accepted HLA-D antigens represent about one third of the total, the remainder have not been identified as yet.

D. RESEARCH DESIGN AND PLANS:

The initial plans for this contract were to follow a sequence of events.

1. Establishment of a contract facility with a laboratory capacity to study the human histocompatibility system and do routine mixed lymphocyte culture testing.

2. Preparation of a panel of homozygous typing cells (HTCs) capable of identifying the common HLA-D specificities.

3. Preparation of panels of primed lymphocyte typing (PLT) cells for use in confirming the results of initial HTC typing, and for use when rapid knowledge concerning an individual's HLA-D type is essential.

4. Cryopreserving and storing these reagents in such quantities that they would allow typing of large numbers of individuals whenever necessary.

5. Identify and quantitate the relative role of each of the major histocompatibility complex genes on clinical transplantation as it relates to graft and donor survival, graft versus host disease and reconstitution of normal immunologic function.

6. In collaboration with Navy and contract facilities identify genetic control of immunologic response in humans.

E. RESULTS

1. Advances in understanding of HLA-D region genetics

We were one of the largest scientific contributors to the Eighth International Workshop held in Los Angeles, February, 1980. During the Eighth Workshop this laboratory worked jointly with the Duke University School of Medicine Immunogenetics group. Georgetown performed all cellular typing and Duke performed all serologic typing.

One hundred fifty individuals including eleven families were typed twice with homozygous typing cells representing all known specificities and all proposed new HLA-D specificities. The families selected were generally large (up to 12 children) and demonstrated segregation of all known HLA-D specificities. In addition, four of the families demonstrated recombination within the HLA region. Including these eleven families, 22 individuals who were known to be positive for the specificities Dw8, 9, 10 and DuB 15 (proposed DRw 9) and numerous previously undefined specificities were typed for.

The Georgetown laboratory submitted several reagents for use in the Workshop. These included homozygous typing cells for the specificities Dw2, 4, 9 and one new specificity designated by the Workshop as DB2.

In addition to typing these individuals for HLA-D, they were serotyped using more than 300 antisera typing for all known and proposed HLA-A, B, C, DR and MB specificities. The findings of the Workshop will be published in a book. Our group made six presentations of findings during the Workshop Conference.

As part of the Workshop, we identified a number of new local homozygous typing cell donors. Two donors for Dw5 and one each for the specificities Dw2, Dw6, Dw9 and the new specificity DB3 were identified. We are the only American laboratory to have identified homozygous typing cells with the specificity Dw9, DB2 or DB3. Some of the findings of our group are:

a) Typing for previously defined HLA-D specificities. The specificities Dw1, 2, 3, 4, 5, 6, 7, and 9 were clearly typed on our panel. Dw8 continued to be difficult to define.

b) Typing for previously defined HLA-DR specificities. HLA-DRw 1,2,3,4,5,7 and 8 could be clearly typed. DRw6 was difficult to define. Two new specificities, DRw12 (corresponding to DuB15 or DRw4x7) and DRw13 (a subgroup of DuB15 called ST) were proposed.

c) HLA-D and DR relationship. The B cell serotype specificities DRw 1-7 generally corresponded to the Dw specificities 1-7. However this correspondence is, at least in part, an artifactual one. During the Seventh International Workshop a large number of investigators felt that HLA-D could, in fact, be defined by the antisera specifically active against B-cells. For example antisera which typed cells expressing the HLA-D specificity Dw1 (as defined by homozygous typing cells) were called anti-DRw1 antisera thus linking the DR and D nomenclature. During the Seventh International Workshop in 1977, Duke and Georgetown and the Naval Medical Research Institute, working together, identified many individuals ...o

could be typed for an HLA-DR specificity but clearly could not be typed for the corresponding HLA-D type. At that time most laboratories felt the discrepancies between HLA-D and HLA-DR type were an artifact of the technical variations in these B-cell serologic HTC and typing systems. In 1978 we presented the first experimental evidence for two independent HLA-D region genes. We used primed lymphocyte typing to demonstrate that HLA-D and HLA-DR act independently in one family. This year we have extended this data to show the independent activity of HLA-D and -DR (see below).

After several years of disagreement among the international HLA typing community, our view has prevailed. Other laboratories have confirmed our initial observations. Further, numerous HLA-D specificities have been found to correspond to single HLA-DR specificities. That is, there are now three HLA-D specificities (Dw2, Dw12 and TB24) which B-cell serotype as DRw2; there are three HLA-D specificities (Dw4, Dw10 and DB3) which serotype as DRw4 and two specificities (Dw6 and Dw9) which serotype as DRw6.

d) New HLA-D specificities. Five new HLA-D region specificities were identified during the Eighth Workshop. These were provisionally designated "DB". DB1 was identified as a HLA-B13 variant of Dw7. DB2, which was submitted by this laboratory, is a HLA-B40 variant of Dw5. DB3 defines a group of cells which types as HLA-DRw4 but fail to type as Dw4. DB4 is an HTC specificity which type some DRw2 cells that fail to type for the Dw2 specificity. This specificity, formerly called DHO, is very prevalent in the Japanese population and

is associated with HLA-B52. DB4 is sufficiently characterized to be given the designation Dw12. Another new cell (TB24) from Sardinia defines a second group of DRw2 "positive" but Dw2 "negative" cells. TB24 is important as it defines the DRw2 positive, Dw2 negative specificity segregating in the family which was used in our initial separation of HLA-D and HLA-DR using Primed Lymphocyte Typing.

e) Relative roles of HLA-D and -DR in leukocyte culture-gene interaction experiments. Although it is now widely accepted that HLA-D and HLA-DR represent distinct gene products, the relative role of these two gene products, and the potential role of other cell surface determinants, in the mixed lymphocyte reaction are not fully explained.

Potentially HLA-D could play a singular role in HTC typing if this gene product acted through the mechanism of suppression. That is, an HTC which carried the specificity Dw1 might prevent (suppress) a resonder cell (the cell being typed) which carried the Dw1 specificity from replicating in a mixed leukocyte culture. Thus the suppression could result in a typing response. This hypothesis was tested in three cell experiments: a) One responder cell; b) an irradiated stimulator which did not share an HLA-D specificity with the responder; and d) an irradiated homozygous typing cell which shared a specificity with the responder. The HTC failed to suppress. This finding has been submitted for presentation at the annual meeting of the American Association for Clinical Histocompatibility Testing (see abstract below).

Thus several cell surface determinants are likely to affect HTC typing. Currently our theory is that both HLA-D and HLA-DR are capable of stimulating in MLC. This is consistent with earlier findings that HLA-D and HLA-DR act independently in primed lymphocyte typing. Second, each HLA-D type is associated with one HLA-DR type. That is, Dw2 is associated with only DRw2; Dw12 is also associated with only DRw2. Note that one DR type may be associated with several HLA-D types. Thus a homozygous typing cell for Dw2 in fact stimulates with both Dw2 and DRw2 and fails to stimulate cells which express both Dw2 and DRw2 HTCs for Dw12 expresses both Dw12 and DRw2 and fails to stimulate responder cells with these same two specificities.

f) Attempts to find antisera which define HLA-D. The failure to identify antisera which define the HLA-D specificities continues to be poorly understood. Cells which express D-DR differences (e.g. DRw4, Dw10) are being used both by the Duke Immunogenetics groups and Georgetown laboratory in an attempt to identify B cell antisera which might differentiate two types of DR bearing cells (e.g. Dw4 + DRw4 vs Dw10 + DRw4). Some of these initial findings were presented at the Eighth International Histocompatibility Typing Workshop Conference (see abstracts below).

g) Statistical evaluation of HTC typing - new approaches. An additional complex problem in HTC typing is the method of data analysis. The method currently used is the "double normalized value" (DNV) which is an arithmetic data conversion that compensates for technical variability in the functional capacity of the many stimulating and responding cells used in

each experiment. Generally a DNV of less than 40 is considered to be a typing response, 40-60 considered an indeterminate result and a value greater than 60 is a non-typing response. The use of fixed cutoff values (40 = + and 60 = -) makes the assumption that only one gene product determines specificity. In order to use the DNV but eliminate the assumption of one gene as the only genetic model, we have developed a method of analyzing DNV's as a continuous variable. This method of using Spearman's Ranked Correlations was presented at the Eighth International Histocompatibility Typing Workshop (see abstract below).

h) Continued implementation of computer techniques. In addition to the statistical developments, capabilities of the in-house Wang 2200 computer system is being developed. Software systems to automatically input, edit, compute and store data on disk files have been developed. A data management system (AIMS) has been implemented for maintaining records of cell specificity identification and inventory control. The computer system is being developed so that information stored for word processing, data management (AIMS), telecommunications (for use on the National Institutes of Health computer system), and our local data entry and editing can be transferred and used in any of these systems.

2. Advances in Primed Lymphocyte Typing.

We have continued to develop primed lymphocyte reagents and expand these reagents with T cell growth factor (TCGF). Reagents for all of the known HLA-D and -DR specificities have been generated. However at this time routine typing for HLA-D region antigens is performed with the B-cell serology and HTC techniques. PLT is not routinely used as most primed cells identify both HLA-D and DR. That is, a cell primed for DRw2 and Dw2 will be stimulated by (type) cells which are DRw2 + Dw2 as well as cells which are DRw2 + Dw12 and cells which are DRw2 + TB24.

We are using two techniques to develop primed lymphocyte reagents which detect HLA-D but not HLA-DR. First, cells which express one DR specificity but a non-corresponding D specificity (e.g. DRw2 + Dw2) are stimulated in the primary phase to a cell sharing DRw2 but expressing TB24. This results in a cell primed to the HLA-D specificity TB24 without DRw2. The second alternative is to establish clones of primed cells. Theoretically, the specificity that these clones recognize should be extremely narrow. At the minimum, one can expect certain clones of primed cells to recognize HLA-D while others generated in the same initial priming cultures would recognize HLA-DR.

3. Development of cloning techniques.

Human T-lymphocytes can be cloned in semi-solid medium by inducing mitogenesis with PHA or culture fluid supernatants (CM) from PHA-stimulated peripheral blood lymphocytes. Furthermore, cell lines from such cultures can be maintained in vitro in the presence of CM. Using these approaches, we are setting up procedures for establishing clonal cell lines from MLC-primed cells

(PLT cells) which hopefully will enable dissection of fine specificities that are recognized in allogeneic reactions. Briefly, cells of selected specificity are first primed in a typical mixed lymphocyte culture, transferred to a semi-solid medium containing CM and plated at low cell concentrations in multi-well plates. Plating in such viscous medium precludes nonspecific cell aggregation as well as localizing proliferating clones. After 5-7 days incubation, clones are removed from positive wells with a sterile pipette and transferred to liquid cultures containing CM and passaged every 2-3 days. Once clonal cell lines are established in sufficient numbers, their ability to recognize subtle yet discrete differences on cells of large typing panels can be assessed.

F. Conclusions from research completed

Using many of the new techniques available to our laboratory we have been able to develop a sophisticated system of accurate histocompatibility typing. Although the system is complex, it is likely that techniques can be developed for rapid large-scale typing which is essential to the transplant community.

G. Proposal for the continuation of this contract.

The initial success and yield of relevant typing data in the HLA field has encouraged us to contine this line of research.

WORK PLAN

a. Continued development of HTC and PLT typing system.

Under this contract we will continue to develop our capabilities to accurately identify and type random individuals. This will be accomplished by the screening of random donors in both assay systems, and the identification of new specificities.

b. Cloning of PLT cells.

During this next year we plan to formally clone the PLT cells from single cells. The expanded clones will be characterized for specificity, surface markers, and capacity to survive in long-term culture.

c. Evaluation of cytotoxic cells in PLT cell pools.

During the generation of PLT cells, killer cells also arise in the mixture. It is possible that such cells may be useful for typing. Work will be done to characterize these cytotoxic cells.

d. Optomization of PLT priming techniques.

Little is known regarding the surface HLA-D region antigens are processed and presented to cells that subsequently react in the MLC. During this year we will begin to investigate the cell types involved in antigen presentation and the means by which to enhance this function. Knowledge of the parameters of priming may allow us to develop assays for many weak and currently undetected antigens.

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ATTACHMENT 1

Human PL Cells Grown as Continued T-Cell Cultures

F. Pappas, G. D. Bonnard, R. J. Hartzman, and D. M. Strong

THE antigens responsible for the mixed lymphocyte reaction (MLR) are controlled by the HLA-D locus of the major histocompatibility complex.[1] Cells sensitized in MLR and cultured for 10 days represent primed lymphocytes (PL) that undergo rapid proliferation to lymphocytes bearing the specific sensitizing HLA-D and DR antigens in a secondary MLR (the PLT test).[2,3] Thus, PL may be used as a rapid method to identify HLA-D region antigens. One major drawback of this technique has been the difficulty in obtaining sufficiently large numbers of PL with a given specificity. An alternate means of generating cellular reagents, active and specific in the PLT, was highly desirable.

Conditioned media (CM) from phytohemaglutinin (PHA)-stimulated leukocytes have been used to maintain long-term cultures of human peripheral blood T cells.[4,5] These cultured T cells (CTC) retain several functions of T lymphocytes and respond in mixed lymphocyte reactions (MLR).

MATERIALS AND METHODS

Both commercial (Associated BioMedics) and CM prepared in the laboratory at NIH was used.[6] RPMI-1640 with 10% fetal bovine serum (FBS), 10% CM, 50 μg/ml gentamycin, and 35 m*M* L-glutamine was used in all experiments.

PL cells were prepared according to the technique of Sheehy et al.[2] HLA-D-homozygous typing cells (HTC) were used as responders and either HTC or HLA-D-heterozygous cells as stimulators.[7] On day 10, PL were either used fresh or cryopreserved according to the method of Strong et al.[8] Frozen-thawed or fresh PL were cultured with CM as cultured T cells (PL-CTC) in 50-ml tissue culture flasks (Falcon, Model 3013, Oxnard Calif.) in concentrations of 5×10^5 cells/ml. Cell counts, using trypan blue exclusion to determine viability, were performed every 2 days. During the culture period, cell concentrations were maintained at $1\text{-}3 \times 10^6$/ml. A portion of the replicating cells was removed at intervals between days 7 and 33 in CM. The cells were removed from CM, allowed to rest 24 hr in RPMI 1640 with 10% FBS, and then frozen in 7.5% dimethyl sulfoxide using a control-rate freezer.

To determine if cells retained specificity, the frozen PL-CTCs and the original primed cells (not treated with CM) were restimulated in a secondary phase with a panel of HLA-D typed heterozygous and homozygous cells. Briefly, 2.5×10^4 responders were cocultured with 5×10^4 stimulator cells in 96-well round-bottom plates (LINBRO MRC 96 TC, Hamden, Conn.) for 48 hr, labeled with 1μCi of ^{3}H-thymidine (2 Ci/m*M*), and harvested after an additional 12 hr using a MASH. Incorporation of thymidine was then measured.

RESULTS

PL-CTCs Developed From Frozen PL Cells

PL cells representing seven established specificities (Dw1–Dw7) previously generated and cryopreserved were thawed and cultured with CM over a period of 1 month. A total of 14 cells, 2 for each specificity, were tested.

Maximum yield from most of the CM-treated cells was reached between 12 and 15 days in culture. Starting with 2.5×10^6 cells, 1 primed lymphocyte (PL5) reached 74.5×10^6 cells by 12 days of CM culture. This was the maximum seen using frozen PL cells. The average increase was 15 times the number of initially cultured cells. Some of the cells (PL10 and PL12) doubled in the first 5 days in culture but then began to decrease. All but

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Table 1. Secondary Phase Stimulation of PL-CTCs From Frozen PLs

	Responder Cells								
Stimulator Panel HLA-Dw Type	PL4 3↓2*	PL4-CTC Day 13	PL4-CTC Day 16	PL6 4↓3	PL6-CTC Day 13	PL6-CTC Day 16	PL5 4↓3	PL5-CTC Day 13	PL5-CTC Day 16
4/4	16,908†	1,274	479	618	302	178	716	—	127
2/4	35,145	6,854	3,242	360	288	391	426	—	141
3/3	390	114	182	20,556	933	119	21,219	—	85
2/2	49,248	15,108	7,750	1,152	265	114	1,493	—	190
4/4	14,342	448	—‡	196	98	84	516	126	84
3/3	2,256	137	—	36,974	1,490	721	44,713	716	149
3/3	3,087	191	156	36,724	1,931	739	29,998	753	337
2/2	48,142	12,067	6,261	1,441	192	299	2,881	—	133
2/4	38,640	8,167	4,044	1,618	353	264	1,655	—	170

*3↓2 represents HLA-Dw3/3 homozygous cell primed to an HLA-Dw2 homozygous cell.

†Values in cpm

‡Not done.

two of the frozen–thawed cells (PL2 and PL13) showed poor survival after 16 days in culture.

Table 1 and Table 2 show the results of six of these cells when restimulated with a panel in secondary phase. In Table 1, PL4 retained its discriminatory ability up to day 16. In fact, this primed cell appeared more selective after expansion with CM. The two HLA-Dw4 panel members giving "false positive" responses with the nonexpanded PL4 do not restimulate the 13 and 16 day cells. PL5 and PL6, however, lost most of their secondary phase reactivity after treatment with CM. In Table 2, primed lymphocytes 7, 8, and 11 demonstrate generally equal or greater selectivity of the CM-treated cells.

PL-CTCs Developed From Fresh PLs

These cells were placed in CM directly from their 10-day primary phase. Starting with 2.5×10^6 cells, 2 of the cells had reached 45×10^6 and the third 32×10^6 by the seventh day in culture. Some of the cells were

Table 2. Secondary Phase Stimulation of PL-CTCs From Frozen PLs

	Responder Cells								
Stimulator Panel HLA-Dw Type	PL7 2↓4*	PL7-CTC Day 13	PL7-CTC Day 16	PL8 2↓2/4	PL8-CTC Day 13	PL8-CTC Day 16	PL11 2↓6	PL11-CTC Day 13	PL11-CTC Day 16
2/2	5,369†	1,459	1,154	2,429	609	608	2,687	849	891
7/7	2,280	637	308	1,967	436	274	1,833	118	173
4/4	23,381	13,328	6,283	38,003	7,592	6,222	4,267	419	423
2/4	10,776	6,151	3,122	26,398	2,399	1,338	872	121	124
6/7	10,052	4,334	—‡	18,267	1,954	1,322	14,249	3,557	2,861
6/8	3,585	1,388	—	1,328	2,627	1,033	32,340	3,387	3,319
5/5	20,039	7,133	—	23,137	6,955	6,492	15,943	2,067	2,062
5/6	13,092	5,049	—	11,311	3,341	2,548	25,202	2,491	2,844
5/7	4,840	—	—	3,519	565	512	602	255	260
4/4	27,565	10,289	5,520	15,170	6,336	4,006	1,634	—	168
2/4	18,311	7,629	3,936	34,514	3,179	1,755	685	—	139
3/3	2,874	—	—	5,917	435	441	6,600	—	516
1/1	7,018	—	—	5,503	1,503	1,593	7,182	—	—
3/7	8,526	—	—	3,798	4,089	3,500	12,085	—	—
6/7	5,772	—	—	7,752	251	225	21,000	2,345	1,349

*2↓4 represents HLA-Dw2-homozygous cell primed to an HLA-Dw4-homozygous cell.

†Values in cpm.

‡Not done.

Table 3. Secondary Phase Stimulation of PL-CTCs From Fresh PLs

Stimulator Panel HLA-Dw Type	Responder Cells: PL1	PL1-CTC Day 7	PL1-CTC Day 22	PL2	PL2-CTC Day 7	PL2-CTC Day 22	PL3	PL3-CTC Day 7	PL3-CTC Day 22
	2x1*			2x7			3x4		
2/2	2,724†	649	1,070	4,315	625	2,698	11,962	3,447	3,875
3/3	7,897	3,184	1,212	5,498	1,393	2,827	1,687	375	878
1/1	24,622	16,984	4,569	3,271	794	2,982	3,821	1,643	2,163
7/7	2,680	1,338	918	14,605	8,113	8,620	4,510	1,433	1,602
4/4	13,217	7,224	6,341	9,464	4,185	2,982	32,658	19,359	10,193
1/3	18,220	9,387	3,278	2,193	362	989	3,306	859	415
1/4	9,985	4,740	4,387	1,544	1,070	2,820	2,778	3,097	2,514
1/8	30,631	23,139	11,114	7,324	3,147	5,567	13,610	5,605	6,685
5/7	11,622	3,614	4,680	19,435	12,815	11,497	13,037	4,046	5,353
3/7	8,978	3,868	1,457	15,554	10,696	9,705	6,900	2,448	2,343
7/7	8,605	2,470	2,043	22,198	11,549	8,329	13,229	5,882	4,047
3/7	10,147	4,382	2,163	21,530	15,368	13,324	6,290	2,121	2,680
2/4	5,810	1,471	964	3,683	1,788	1,188	11,585	5,686	2,979
2/7	2,132	971	1,000	2,828	718	2,704	6,584	1,191	2,279
4/5	12,756	7,919	6,891	13,389	11,339	10,455	20,084	11,651	8,437

*2x1 represents HLA-Dw2-homozygous cell primed to an HLA-Dw1 homozygous cell.

†Values in cpm.

frozen and others allowed to continue. Unfortunately, the CM that was being used up to this point was switched to another lot that functioned poorly. It was decided to stop the cultures at day 22, since the cells began to decline in numbers.

The results of *secondary phase stimulation* of the unexpanded day-7 and day-22 CM-treated cultures are seen in Table 3. Again, the treated cells, in addition to maintaining their specificity, show in some cases better discrimination between positives and negatives, although the overall cpm is somewhat decreased.

DISCUSSION

Results from this study show that functional PL can be expanded using CM. These cells maintain specificity and in some cases even showed increased discrimination. However, several problems were encountered. In general, the absolute cpm obtained from PL-CTC was decreased, although typing responses were easily detected. In the initial experiment using CTC generated from frozen–thawed PL, cell viability became a problem beyond 14 days in culture with CM. Additional experiments with fresh (day 10) PL-CTC suggested that this may have simply been a technical problem and that these cells can be grown in a manner comparable to that described for other CTC[5,6] with starting cultures of 5×10^6 cells developing into 2×10^8 cells within 14 days. Further experiments are planned to answer the more important question as to whether 16–30-day-old PL-CTC lose their specificity.

Thus, large numbers of PL primed for each of the HLA-D region specificities can be generated using the PL-CTC system. This will allow for a more practical application in the transplant situation, since adequate numbers of cells can be made available for typing more individuals. Further, the ability to maintain continued growth of primed cells in vitro will permit numerous other studies, such as characterization of the idiotypic nature and diversity of receptors for cloned PLT cells.

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ATTACHMENT 2

POSSIBLE NEW HLA-D SPECIFICITIES

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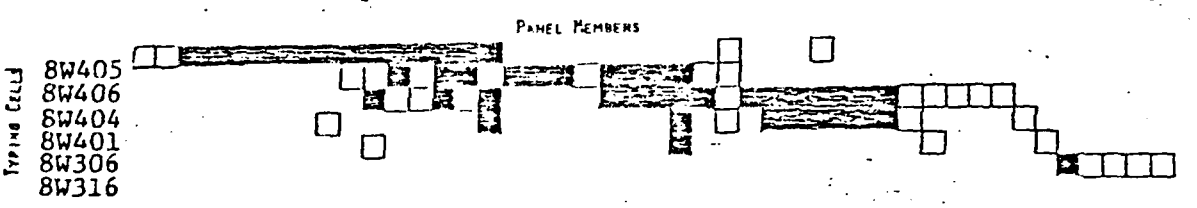
A panel of 64 unrelated responders was typed in primary MLC using HTC's submitted for the 8th International Workshop. Positive responses were determined by the geometric means of DNV's 40 (black boxes) and DNV's 60 (open boxes) from duplicate experiments. After excluding typing cells corresponding to known HLA-D specifities and those cells which could not be classified into a restricted number of groups due to high reaction frequencies, the following groups were obtained:



Such a pattern of response may indicate new specificities which have not been described previously. Preliminary analysis revealed a good correlation between panel members typed by 8W316 and sera containing DRw(4x7) specificity. Additionally, 8W316 failed to type 9/9 Dw4 and 4/4 Dw7 individuals although it did detect 3/3 DRw(4x7) and 2/6 Dw10 panel members. Such a pattern of reactivity may be due to the complex relationships which exist among DRw4, Dw4 and Dw10 specificities and the proposed Dw(4x7) typing cell. It is hoped that further analysis of matched typing responses by newly described serologic specificities and submitted HTC's will corroborate these findings.

A panel of 64 unrelated responders was typed in primary MLC using HTC's submitted for the 8th International Workshop. Typing cells corresponding to known HLA-D specifities and those which could not be classified into a restricted number of groups due to high reaction frequencies, were excluded. Rank correlations were determined according to Spearman. Several discrete and overlapping groups were identified which may characterize new antigens generating proliferative responses in primary MLC. Double normalized values (DNV's) less than 40 (solid boxes) were considered positive responses while DNV's greater than 40 but less than 60 were considered doubtful but positive (See figure) in duplicate experiments.

Analysis revealed a good correlation between panel members typed by 8W316 and sera containing DRw(4x7) specificity. Additionally, 8W316 failed to type 9/9 Dw4 and 4/5 Dw7 individuals although it did detect 3/3 DRw(4x7) and 2/6 Dw10 panel members. Such a pattern of reactivity may be due to the complex relationships which exist among DRw4, Dw4 and Dw10 specificities and the proposed Dw(4x7) typing cell. Workshop cells 8w401 and 8w406 do correlate with known HLA-D/DR specificities although to a marginal degree. That is, 8w401 seemed to type both Dw2 and DRw2 cells while 8w406 types Dw6 and Dw8 cells but reacted only with serum 8w55, yet both cells appear to type other individuals as well. A number of different serum correlations were noted for 8w401. These relationships might be accounted for by antigenic similarities between families of antigens or new specificities. While the final significance of such patterns remains to be resolved, it is hoped that further analysis of matched typing responses by newly described serologic specificities and submitted HTC's will elaborate and elucidate these findings.

Comparison of Unknown Panel with HTC's of HLA-Dw1-11 Specificity
Using Means of Spearman's Correlation Coefficients

HLA	8w405	8w406	8w404	8w401	8w306	8w316
Dw1	0.220	0.065	0.175	0.160	0.225	0.025
Dw2	0.025	0.026	0.345*	0.380*	-0.165	0.007
Dw3	-0.027	-0.124	0.025	0.039	0.108	0.004
Dw4	0.303	-0.020	0.191	-0.008	0.022	0.058
Dw5	0.110	0.008	0.045	0.032	-0.081	-0.068
Dw6	0.184	0.396*	0.060	0.108	-0.044	-0.155
Dw7	0.215	0.022	0.015	-0.028	0.142	-0.075
Dw8	0.015	0.430*	0.232	0.201	0.231	-0.124
Dw9	0.060	0.232	0.029	0.063	0.205	-0.253*
Dw10	0.145	0.144	0.222	0.066	0.177	0.100
Dw11	0.179	-0.116	0.035	-0.071	0.144	0.058

*$P < 0.05$

Comparison of Cells of Unknown Specificity Using Spearman's Correlation Coefficients and Probabilities

$r_s \backslash p$	8w405	8w406	8w404	8w401	8w306	8w316
8w405	-	0.007	0.312	0.011	0.918	0.618
8w406	0.332	-	0.001	0.002	0.050	0.023
8w404	0.128	0.400	-	0.0001	0.023	0.609
8w401	0.317	0.389	0.648	-	0.581	0.995
8w306	0.013	0.246	0.283	0.070	-	0.835
8w316	-0.064	-0.283	0.065	-0.001	0.027	-

Significant Associations of Unknown Cells with B-Lymphocyte Serologic Specificities*

	Param.	r	Non-Param.	r
8w405	DRw6	0.51	DRw6	0.59
	DRw7	-0.32	--	--
	8w67	0.41	8w67	0.40
	9(1,2,6)MB1	0.39	--	--
	12(3+6)	0.35	12 (3+6)	0.45
	55 IA76	0.43	55 IA76	0.56
8w406	8w55	-0.35	--	--
8w404	DRw2	0.57	DRw2	0.44
	DRw6	-0.34	DRw6	-0.34
	8w55	-0.27	--	--
	8w64	-0.30	8w64	-0.35
	17-15	0.29	17-15	0.36
	52 WMC	-0.29	52 WMC	-0.38
	54 Dw9	-0.78	54 Dw9	-0.50

	Param.	r	Non-Param.	r
8w401	DRw2	0.46	DRw2	0.61
	DRw4	-0.43	--	--
	8w64	-0.48	8w64	-0.41
	51 MCL	-0.46	--	--
	53 MB3	-0.26	53 MB3	-0.38
8w306	DRw5	-0.32	--	--
	8w61	-0.28	--	--
	40S	-0.28	--	--
	9(1,2,6)MB1	-0.32	9(1,2,6)MB1	-0.45
8w316	DuB29(wIA8)	-0.51	DuB29(wIA8)	-0.4
	15(4x7)	0.52	15(4x7)	0.5
	54 Dw9	-0.29	--	--
	--	--	9(1,2,6)MB1	-0.4

*$p < 0.05$ for all comparisons presented.

Serologic Reactions of Cells Typed by Sw316

Dw4

	+	-
+	0	5
-	9	31

$\chi^2=3.16$, $P=0.0755$

Dw7(w11)

	+	-
+	1	4
-	4	36

$\chi^2=0.007$, $P=0.933$

DRw4

	+	-
+	2	3
-	9	31

$\chi^2=0.078$, $P=0.780$

DRw7

	+	-
+	1	4
-	8	32

$\chi^2=0.352$, $P=0.553$

DuB15(4x7)

	+	-
+	3	0
-	0	42

$\chi^2=30.4$, $P=3.56\times10^{-8}$

Dw10

	+	-
+	2	3
-	4	36

$\chi^2=1.35$, $P=0.245$

HLA Specificities of Responder Panel

Typing Cell	Panel Member	HLA Specificity					
		A	B	w4/w6	Cw	Dw	DRw
8w405	1	2,w24	15,w22	6	3	9	6
	2	w24,w26	40,w51	4,6	2	-	6
	3	2,w24	8,w44	4,6	5	3,4	3,4
	4	2	w35,w51	4,6	4	5,9	5,6
	5	w28,w33	7,17	4,6	6	-	6
	6	2	40,w41	6	3	5,6	5,6
	7	2,w23	17,w49	4	3	-	4,6
	8	3,11	w41,w52	4,6	-	4,6	4,7
	9	3,11	40,w51	4,6	2	5,6	5,6
	10	3,w33	7,14	6	-	2	2,7
	11	26,w31	w44,w49	4	5	10	4,6
	12	1,3	7,8	6	-	1,3	1,3
	13	2,w33	17,w41	4,6	3	3	3,6
	14	1,2	27,40	4,6	2,3	3,6	3,6
	15	3	14,w35	6	4	4	4,6
	16	w23,25	7,40	6	-	2	2
	26	2,3	7,8	6	-	2,6	2,6
	30	3,w24	14,w35	6	4	1	1,2
8w406	10	3,w23	7,14	6	-	2	2,7
	11	26,w31	w44,w49	4	5	10	4,6
	12	1,3	7,8	6	-	1,3	1,3
	13	2,w33	17,w41	4,6	3	3	3,6
	14	1,2	27,40	4,6	2,3	3,6	3,6
	15	3	14,w35	6	4	4	4,6
	16	w23,25	7,40	6	-	2	2
	17	2,w24	13,40	4,6	3	-	6
	18	2,w24	w35,w38	4,6	4	9	-
	19	2,29	w34,40	6	3	8,9	4
	20	1,2	17,w51	4	1,6	11,8	7
	21	1,28	37,w44	4	5,6	2,5	2,5
	22	w32,w33	17,w35	4,6	4,6	-	1
	23	29,w31	40,w44	4,6	2	5	5,7
	24	2,11	17,27	4	2	2	7
	25	2,w24	27,w44	4	2,5	4,5	4,5
	26	2,3	7,8	6	-	2,6	2,6

HLA Specificities of Responder Panel (continued)

Typing Cell	Panel Member	HLA Specificity					
		A	B	w4/w6	Cw	Dw	DRw
8w404	11	26,w31	w44,w49	4	5	10	4,6
	12	1,3	7,8	6	-	1,3	1,3
	13	2,w33	17,w41	4,6	3	3	3,6
	14	1,2	27,40	4,6	2,3	3,6	3,6
	15	3	14,w35	6	4	4	4,6
	16	w23,25	7,40	6	-	2	2
	21	1,28	37,w44	4	5,6	2,5	2,5
	22	w32,w33	17,w35	4,6	4,6	-	1
	23	29,w31	40,w44	4,6	2	5	5,7
	24	2,11	17,27	4	2	2	7
	25	2,w24	27,w44	4	2,5	4,5	4,5
	27	2,26	w38,w41	4,6	-	10	4
	28	2,26	8,w52	4,6	-	3	2,3
	29	2,w30	17,w45	4,6	-	2	2
	30	3,w24	14,w35	6	4	1	1,2
	31	3	7	-	-	2,8	2
	32	w30	7,w53	4,6	4	2	2,5
	33	1,3	7,8	6	-	2,11	2,7
	34	2,11	7,27	4,6	1	2,3	2,3
	35	w24,w31	14,15	6	3	4,7	4,7
	36	2,29	37,w45	4,6	6	3,11	3,7
	37	3,w24	7	6	-	2	2
	38	2,w30	13,w41	4,6	6	1,3	1,3

HLA Specificities of Responder Panel (continued)

Typing Cell	Panel Member	HLA Specificity					
		A	B	w4/w6	Cw	Dw	DRw
8w401	10	3,w33	7,14	6	-	2	2,7
	16	w23,25	7,40	6	-	2	2
	24	2,11	17,27	4	2	2	7
	26	2,3	7,8	6	-	2,6	2,6
	28	2,26	8,w52	4,6	-	3	2,3
	30	3,w24	14,w35	6	4	1	1,2
	31	3	7	-	-	2,8	2
	32	w30	7,w53	4,6	4	2	2,5
	33	1,3	7,8	6	-	2,11	2,7
	34	2,11	7,27	4,6	1	2,3	2,3
	39	3,w31	7	6	-	2	2
8w306	11	26,w31	w44,w49	4	5	10	4,6
	24	2,11	17,27	4	2	2	7
	35	w24,w31	14,15	6	3	4,7	4,7
	40	1,3	7,w35	6	4	2	2
8w316	41	1,11	8,15	6	1	3	3
	42	26,w30	w38,w44	4	-	11	7
	43	25,26	18,w38	4,6	-	5,10	4,5
	44	28,w33	w45	6	3	-	1
	45	1,2	15,w51	4,6	3,4	6,10	4,6

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Summary of the First International Workshop on Human Primed LD Typing

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It is now recognized that the proliferative response in mixed leukocyte cultures (MLCs) is due primarily to the recognition by responder cells of lymphocyte defined (LD) antigens on the surface of stimulator lymphocytes. The currently known LD antigens are encoded by a locus within the major histocompatibility complex (MHC), located on the 6th chromosome, which has been termed the HLA–D locus as distinguished from the serologically defined (SD) closely linked HLA–A, –B and –C loci. There has been considerable interest in the ability to determine the genetic polymorphism of the HLA–D locus and nearby regions. Recently, a technique has been described which provides a powerful tool for the examination of this genetic polymorphism. The test is called the primed LD typing [or primed lymphocyte typing (PLT)] test and is based on the observation that lymphocytes can be primed with allogeneic lymphocytes *in vitro* (Hayry & Anderson 1974, Fradelizi & Dausset 1975, Zier & Bach 1975). When these primed cells are restimulated with the antigens to which they were originally sensitized, a vigorous secondary response is observed. It was determined that human lymphocytes primed *in vitro* to cells differing by a single HLA haplotype (or HLA–D region determinant) and incubated for 9–14 days can be restimulated to respond in a secondary manner by cells carrying the same HLA–D region antigens present on the initial sensitizing cell. The unknown cells which give rise to such a secondary response, therefore, possess similar if not identical LD antigens to the priming (reference) cell. This procedure has led to a method for typing of surface antigens encoded by the HLA–D region (Hirschberg et al. 1975, Sheehy et al. 1975, Crosier et al. 1977b). This assay is currently being used in various laboratories around the world. Members of 17 laboratories participated in a conference on PLT typing held at the Naval Medical Research Institute, Bethesda, Maryland. This is a summary report from that workshop.

I. Technical Aspects

a) Methods

Although numerous modifications of the basic method have been successfully employed, all current methods basically incorporate the technique initially described by Sheehy & Bach (1976).

Primary sensitization: Responding lymphocytes and mitomycin-treated or X-irradiated stimulating cells are usually co-cultured in 20 ml of tissue culture medium in 50 ml tissue culture flasks (Falcon Model 3013) standing upright. However, primed cells have been produced using as little as 6 ml medium and 2×10^6 responder and

stimulator cells in tissue culture tubes, or as much as 200 ml of medium with 400 x 10^6 responder and stimulator cells in 2 liter roller bottles.

The primed cells are harvested on days 9–14; some laboratories add or exchange media during incubation. Cell yields are usually equal to the number of responder cells placed in culture, however they may vary from less than half to four times the number of initially cultured responders.

Secondary stimulation: Primed lymphocytes are dispensed into V-bottom or round-bottom 96-well microtiter tissue culture trays for secondary stimulation. Generally, $12.5-100 \times 10^3$ primed responding cells (usually 25×10^3) are cultured with $12.5-200 \times 10^3$ mitomycin-C treated, X-irradiated or untreated fresh stimulating lymphocytes (usually 50×10^3) in a total volume of 0.15–0.2 ml of tissue culture medium per well. Secondary responses are assessed after incubation for 24–72 h at 37°C in a 5% CO_2 atmosphere, by pulsing with 1–2 μCi ^{3}H–TdR (2–60 Ci/mM) per well. Six to 18 h after pulsing, the cells are harvested on filter paper using a sample harvester (Hartzman et al. 1972, Hirschberg et al. 1975) and the radioactive incorporation determined with a scintillation counter.

Tissue culture media: RPMI 1640 buffered with bicarbonate and HEPES (25 mM) is used by most laboratories. The medium is usually supplemented with 5–20% pooled human plasma or serum. Antibiotics, usually penicillin (100 u/ml) with streptomycin (100 μg/ml) or gentamycin alone (50 μg/ml) are also added to the media, as well as fresh L-glutamine.

b) Interpretation of results

Those populations of stimulating cells that induce a secondary response of similar magnitude to the primary stimulating (reference) cell are considered "positive." Those cells that induce proliferation comparable to that stimulated by the primary phase responding individual (control) are considered "negative." In reality, the great majority of stimulating cells induce secondary responses greater than the negative control but less than response to the reference cell. These intermediate responses produce much of the problem in analyzing PLT data. It was felt that intermediate level stimulation may reflect either of two phenomena. First, it could reflect a partial antigen sharing or cross-reactivity by determinants on the test stimulator with that of the reference cell. Second, these intermediate responses might represent "accelerated primary responses" to LD antigens not recognized on the initial stimulator, presumably mediated by responding lymphocytes that had survived in an inactivated state for the 9–14 days of primary sensitization. It was concluded that these two possibilities were not mutually exclusive and that no clear evidence refuting either theory had yet been produced.

c) Technical modifications

Several technical modifications were suggested to enhance the "discriminatory" ability of secondary cultures. Hirschberg and Thorsby added 2-mercapto-ethanol, producing by this technique relatively weak secondary responses, but producing a greater separation of apparent stimulatory ("positive") and non-stimulatory ("negative") secondary responses. Wank reported on the use of low numbers of primed cells (12.5×10^3) in the secondary phase

culture. The use of a decreased number of responding cells appeared to lower the "non-specific" restimulation. Mawas, however, tried the same "titration" approach without influencing the discriminatory ability of these cells. Repeated priming in MLC has been attempted by several groups. Crosier found that one could markedly increase the cell yield by repeated stimulations with a single stimulating individual and occasionally show an increased specificity of these multiply primed cells (Crosier et al. 1977a). Numerous schedules for harvesting of the secondary phase cultures were assessed. Some groups reported greater discrimination by early (24 h) secondary phase harvesting, while others reported no consistent improvement in early versus late (48–72 h) harvesting. Late harvesting offers some technical advantage in that generally higher cpm are obtained, reducing radioactive counting error; however, early harvesting may produce greater selectivity. The problem of identifying the optimum duration of the secondary phase culture is complicated by certain cell combinations which produce peak responses at 24 h, while others do not peak until 72 h of culture. Reinsmoen has used multiple harvesting intervals to avoid the problem of optimal secondary culture duration. No consensus of opinion was obtained.

Several groups have attempted to select for those lymphocytes actively responding in the primary culture. The Paris group (Fradelizi et al. 1977) has separated the theoretically committed blasts from the small lymphocytes on 1G sedimentation gradients during maximum proliferation of the primary phase culture (5–7 days). The separated cells were further cultured for several days and then harvested. The blast cell derived population showed discriminant potential was not improved. On the nant specificity upon secondary challenge, although the discriminatory potential was not improved. On the other hand, the small lymphocyte isolated population from the primary phase, when challenged in the secondary phase, showed no specificity. These data indicate that the blast cells are the progenators of the cells that give rise to discriminatory PLT responses. Rimm and Bach, using a similar unit gravity separation of blast cells, allowed the blast cells to revert to small lymphocytes. They presented preliminary data consistent with increased discrimination with the separated cells as compared with nonseparated cells.

In efforts to determine similarities and/or differences between the responder cells in primary MLR from those in the secondary MLR, Hartzman, Pappas and Sell used a heterologous cytotoxic antiserum against human T cell specific antigen (HTLA). No MLC reactivity was seen when responder cells were pretreated with this antiserum and guinea pig complement prior to primary culture. Interestingly, pretreatment of the primed lymphocytes with an amount of antiserum which completely abolished the primary MLR was neither cytotoxic to primed lymphocytes nor did it alter the secondary proliferative response. Furthermore, treatment of PLT cells with this antiserum did not reduce the so-called nonspecific activation of these cells. Lack of suppression of nonspecific response by anti-HTLA treated cells lends support to the theory that low- and middle-level PLT reactivation is not due to a rapid primary MLR from unprimed lymphocytes in the secondary phase of the PLT.

As a practical approach to generating large numbers of primed cells, a cooperative study by Yunis and Bach was performed to look at priming with lymphoblastoid cell lines generated from homozygous typing cells (Reinsmoen et al. 1977). Such cell

lines are a virtually limitless source of HLA–D homozygous cells, but have the associated difficulty of inducing autostimulation of normal lymphocytes from the cell line donor. In the primary phase PLT, unrelated responding cells were stimulated with homozygous lymphoblastoid cell lines, becoming primed to both *HLA–D* and the "autostimulating determinant". Secondary responses of these cells were tested with a panel of normal stimulating lymphocytes, thus testing only for restimulation by the histocompatibility antigens. Results from PLT cells primed initially with lymphoblastoid cells were highly correlated with results from cells primed with normal homozygous cells.

The issue of "autostimulatory" antigens on lymphoblastoid cell lines was studied in a tumor model with PLT by Reinsmoen. Some Leukemia cells (both ALL and AML) were shown to stimulate weak proliferative responses of either autologous remission lymphocytes or lymphocytes from MHC identical siblings. Restimulation of these cultures with autologous and allogeneic leukemia cells were performed. Secondary response was stimulated by some but not all leukemic cells, suggesting that the PLT technique may provide a means for identifying the specificity of a system of leukemia-associated antigens distinct from the recognition of allogeneic histocompatibility specificities. However, the apparent specificity for subclasses of leukemic cells could also be explained by "self-modification" (possibly by a viral antigen) with secondary phase restriction requiring both histocompatibility determinants and the blast or viral antigen for secondary stimulation.

d) Data analysis

Three new methods were advocated for PLT data analysis. Franks & Bradley (1977) described a method based on the calculation of error estimates. First, Log_e transformation of the data was performed in order to eliminate the difference in variance associated with low and high secondary response cpm. The transformed data were then subjected to a three-way analysis of variance test. This gave a residual mean square value which was used in the calculation of a standard error of group means. A series of least significant differences was then calculated using a series of critical values obtained from Harter's tables (Harter 1960). Critical values for a given number of means at the appropriate degrees of freedom were then multiplied by the standard error of group means to give least significant differences. These least significant differences were then used to perform multiple range testing (Duncan 1955). Each range of restimulation responses with a given responder could be objectively divided into clusters, the members of which were not significantly different at a given probability level.

A second method was proposed by Rubenstein (Rubenstein et al. 1975). This new method was a further development of Piazza's cluster analysis (Piazza & Galfré 1975), which in turn was based on Fisher's method for discriminating analysis (Fisher 1936). By this method, each group of values (beginning with a group of just one value) in a series of responses was compared with each other group of that series. In principle, the analysis was based on an analysis of variance by which the sum of squares value realized an optimum.

A third procedure was proposed by Sheehy (1978). Responses were clustered either into one group with low cpm (the majority) or into a second group with intermediate or high cpm. The low group

appeared to be almost normally distributed. The working model proposed was that the low cpm group clearly did not share any of the LD priming determinants with the reference cell. Essentially the method was performed by grouping the lowest 1/3 of secondary responses and eliminating any high values identified by a test of kertosis. If none of the values fell outside the normal group, the next highest value was added and the kertosis value recomputed. This process of progressively adding the next highest value was continued until a value outside the major low cpm group was identified. All values which fell outside the low group were said to share at least some specificities with the initial priming (reference) cell.

There are a number of other methods currently in use. One such technique is primarily based on the double blind scoring of results using arbitrary division of results into clusters by two independent observers. Relatively high secondary stimulation is considered to demonstrate sharing of specificity between the reference cell (primary stimulator) and secondary stimulator, and conversely low stimulation represents lack of specificity sharing. A second technique, introduced by Thomsen, is based on performing experiments using a number of primed cells and secondary stimulators in a matrix (Thomsen et al. 1976). Each value is normalized to the maximum response of each primed cell followed by normalization to the maximum for each stimulator, and the normalized values are grouped as high- or low-level responses.

Although each method appears promising, extensive testing of the statistical methods with PLT data is necessary. A clearer understanding of the genetics of the PLT response is likely to be necessary before any one method of analysis becomes accepted.

II. Genetics

It is very difficult to develop a sound method of data analysis without a thorough understanding of the genetics of the system. Equally difficult is the problem of defining the genetics of such a complex system without an unbiased method of defining positives and negatives. Nonetheless, certain fundamental principles are evolving.

In general, it was suggested that the PLT discriminates at least three different genetic systems: (1) HLA–D region determinants, perhaps recognizing the same determinant as defined by homozygous typing cells; (2) determinants within the MHC, but probably separate from the HLA–D region; and (3) determinants segregating independently of the major histocompatibility complex. Many laboratories were able to demonstrate methods of priming that resulted in PLT typing, producing very high correlations with HLA–D typing by homozygous typing cell methods.

A number of methods were shown to be useful for generation of these HLA–D associated primed cells. First, priming can be performed with lymphoid cells from family members where primary responder and stimulator share one major histocompatibility complex haplotype. To attain HLA–D associated specificity in family typing, PLT combinations are generated and a preliminary screen against a random panel is performed to identify those combinations where highly discriminant PLT cells are generated. About 1/3 of the PLT cells produced by intrafamilial combination are highly discriminant cells

(Sondel & Bach 1977). However, 2/3 of the primed combinations are discarded, as they produce relatively high secondary responses (when challenged in secondary phase) to many individuals and do not give clearly separable (discriminant) high- and low-level responses. The reason for this lack of discrimination in 2/3 of PLT cells generated by one haplotype priming may be due to either additional MHC loci, non-MHC genes, or a complexity of the HLA–D gene products. The use of multiple combinations (PLT cells generated in a number of families) which identify a single cluster of individuals who share an HLA–D determinant is helpful in eliminating the non-HLA–D effects in PLT typing.

A number of other priming techniques, which take advantage of known HTC typing, have been used successfully to generate PLT cells apparently specific for HLA–D determinants. Various combinations of homozygous typing cells (HTC) and HLA–D typed heterozygous cells have been used in stimulator-responder pairs attempting to generate cells primed against a single HLA–D difference (Hirschberg et al. 1975, Thomsen et al. 1976, Hartzman et al. 1977, Jaramillo et al. 1977, Reinsmoen et al. 1977). Although many of these primary combinations produce useful cells, the most consistent correlations with HTC typing occurred when both responder and stimulator cells were HTCs. In fact, nearly all PLTs generated solely with HTCs are highly discriminant and specific for the appropriate HLA–D type. Of interest, the use of HTCs as responder cells and heterozygous cells sharing one –D specificity as stimulators produced PLT cells which were frequently as specific as those generated with the use of HTCs as both primary phase responder and stimulator. However, in some cases, the HTC-heterozygous combinations were restimulated by cells not bearing the appropriate HLA–D type, suggesting priming to more than one specificity. The success of generating PLT responses, which were highly correlated with HTC typing where HTCs were used in the priming phase, suggests that these HTCs may well have gene restrictions in addition to HLA–D homozygosity which allows their successful use both in primary MLR typing and PLT. Thus, homozygous typing cells may be initially selected on the basis of both homozygosity at HLA–D and restriction on non-HLA–D stimulating specificities.

Wank presented a method of intra-HLA–D group priming in which two HTCs of the Dw4 group were sensitized to each other. By neutralizing the responses to the HLA–D locus, non-HLA–D determinants could be identified (Wank et al. 1978). Suciu-Foca and Rubenstein presented PLT data from families serotyped for HLA–A, –B and –C and typed for HLA–D by the HTC method. Priming was performed either by stimulating lymphocytes from one individual with a second, unrelated individual who differed by a single HLA–D specificity or intrafamilial priming. In these studies, some individuals who clearly did not possess the appropriate HLA–D type for secondary stimulation and did not share an HLA–D type with the reference cell were able to induce a secondary response similar to that induced by the reference cell. However, other family members who shared this MHC haplotype did not cause secondary stimulation. Thus, it appeared that at least one gene segregating independently of MHC is capable of causing both cell priming and secondary response. These studies postulate genetic loci independent of the MHC affecting the

PLT; however, the initial data could be explained by a great deal of complexity of a single genetic region.

A number of studies were carried out to determine gene dose effect in PLT. A cooperative study between the Madison and Copenhagen groups where heterozygous cells were primed with homozygous cells demonstrated greater secondary stimulation by homozygous cells than heterozygous cells with the appropriate HLA–D specificity (Bach et al. 1976). However, this gene dose effect was not confirmed by HTC–HTC or HTC–heterozygote priming in studies at the Naval Medical Research Institute or University of Tübingen (Jaramillo et al. 1977). In addition, family studies by Bradley and by Suciu-Foca and Rubenstein did not demonstrate gene dose effect in PLT.

B cell alloantigens: As the understanding of B-cell alloantigens was still preliminary, generalizations of the relationship between these specificities and PLT could not be made. However, Sasportes and Thorsby were both able to demonstrate strong associations between specificities defined by PLT, HTC and B-cell typing; and Sasportes reported a closer association between PLT and B-cell serologic typing than HTC typing. Hirschberg and Thorsby had used sera which were highly associated with HLA–D typing by HTC and PLT methods and found them capable of specifically inhibiting the PLT secondary phase stimulation of challenge cells known to share HLA–D specificity with the reference cell (Hirschberg et al. 1977).

Comment

The genetic interpretation of third party restimulation is still hampered by technical and biological difficulties as well as a lack of uniform method of data analysis. Methods of priming large uniform batches of PLT cells must be developed. The intricacies of cell concentrations and timing of both primary and secondary phases must be better understood. Much is still needed in the understanding of the fundamental cell–cell interactions leading to *in vitro* priming. An optimum method of data analysis must be developed, but the analysis may have to wait until there is a more thorough understanding of the basic genetics and cellular interactions. A great deal has been defined since the introduction of PLT in humans, yet a great deal more needs to be known before the enormous value of *in vitro* cell priming can be fully realized.

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